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Immunologic aspects of liposomes: presentation and processing of liposomal protein and phospholipid antigens

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Abbreviations: APC, antigen presenting cell; CTL, cytotoxic lymphocyte; IL-1, interleukin-1; IL-2, interleukin-2; MHC, major histocompatibility gene complex; PCC, pigeon cytochrome *c*; Th, helper T lymphocyte.

I. Introduction

I-A. Immunological presentation of protein antigens

In recent years numerous insights into the cellular mechanisms for initiation of the afferent and efferent

limbs of the immune response have led to development of exciting theories in a new field that has come to be known as 'antigen presentation' [1–8]. This field encompasses the cellular mechanisms and processes by which antigenic information is received and translated by the immune system to generate an immune response. Highly sophisticated and useful theories of antigen presentation have been proposed by several laboratories based on research in which virtually all of the investigations were performed with protein or peptide antigens. Many theories even include a requirement for partial degradation of proteins as a vital theoretical element in the basic concept of antigen presentation. One of the major purposes of this review is to describe various ways in which liposomes have been used as models or tools for studying membrane-associated mechanisms that play a role in the induction of immune responses to protein antigens. Liposomes containing protein or peptide antigens have played an important role as models in the development of theories of interactions of membrane-associated protein antigens with antigen presenting cells (APCs) (see Ref. 9 for earlier review).

I-B. Immunological processing of lipid antigens

One of the areas in which theories of antigen presentation are still incomplete is in a theoretical explanation of the mechanisms by which antigens that contain neither protein nor peptide constituents, such as phospholipids and cholesterol, are presented to the immune system. Phospholipids and cholesterol were among the earliest and most highly purified antigens in the field of immunology and, although interest in these antigens has been cyclical, they have been intensively studied since the beginning of this century [10–16]. The development, more than 85 years ago, of a successful serologic test for syphilis [10], in which the components of the syphilis assay consisted of cardiolipin, lecithin and cholesterol, stimulated considerable interest in the immunology of lipids.

One would think that the immunological characteristics of seemingly simple lipid compounds, and the relationships and interactions of lipids with the immune system that have been described in thousands of articles would be well understood from an immunological standpoint by now. However, despite the early development of antisera that appeared to react independently with lecithin, cholesterol, and cardiolipin, particularly by German scientists in the 1920s and 1930s, the ability of these lipid compounds to serve as antigens has generated controversy through the years [12,16].

From the standpoint of an immunologist working in the field of antigen presentation, the argument could conceivably be made that phospholipids and cholesterol cannot be antigenic and specific antibodies to them cannot be induced because of the following reasoning: (a) these compounds lack peptides; (b) because of the lack of peptides they cannot be immunologically presented by APCs according to current presentation paradigms; and therefore (c) because of lack of presentation they cannot stimulate a specific immune response. Although it is highly likely that a mechanism does exist for immunological presentation of lipid antigens, because of the distinct differences between protein and lipid antigens the term 'processing' of lipid antigens is substituted for 'presentation' in technical discussions of immune responses to lipid antigens in this article. Later sections in this review will describe background information and current understanding of antibodies to liposomes and lipid bilayer lipids and non-lipid bilayer lipids, and the implications that such antibodies have for theories of antigen presentation. The implications that antibodies to lipids have for certain concepts of autoimmunity will also be addressed.

II. Liposomal modeling of immunologic presentation of protein antigens

II-A. Theories of antigen presentation

In the immune system specialized cells known as antigen presenting cells (APCs) (also called accessory cells) provide mechanisms both for initial intracellular processing of protein antigens and for causing interactions with T lymphocytes that ultimately lead to specific humoral or cellular immune responses.

Recognition of an antigen by T lymphocytes usually occurs only when the antigen (or a partial degradation product) is 'presented' on the surface of an APC in a defined complex with genetically homologous forms of glycoprotein recognition factors known as major histocompatibility gene complex (MHC) molecules (Fig. 1). An APC can express either class I or class II MHC molecules on its surface. Although all nucleated cells can express class I MHC, only a few cells have the capacity to express class II MHC (also known as Ia antigen). It is believed by most investigators that the latter cells include macrophages, B lymphocytes, and Langerhans and dendritic cells of skin and lymphoid organs.

Current evidence suggests that at least two distinctive categories of antigen may be recognized by APCs and presented to T lymphocytes: extracellular (exogenous) antigens (such as bacteria or other foreign cells or proteins) that are phagocytosed or endocytosed or otherwise taken up by APCs, and intracellular (endogenous) cytoplasmic antigens (such as viruses or tumor antigens). Either category of antigen may lead to presentation with class II MHC as part of an initial

immune response resulting in the induction of antibodies or cytotoxic T lymphocytes (CTLs). The second type of antigen mentioned above (endogenous antigen) may also lead to presentation with class I MHC as part of an effector mechanism process that leads to recognition and killing of the affected cells by CTLs.

In contrast to these T cell-dependent antigens, some antigens, particularly certain carbohydrate or lipid-associated haptens, interact with cells of the immune system in the absence of T cells. These T-independent antigens, which ordinarily generate IgM but not IgG antibodies, lack the ability to be processed by the T cell-dependent antigen presentation machinery of APCs that has been described for protein and peptide antigens. However, it was recently reported that a T-independent antigen in liposomes was converted to a T-dependent antigen by simultaneous inclusion in the liposomes of a peptide sequence (from a hemagglutinin protein of influenza) that is recognized by helper T lymphocytes and CTLs [17]. This demonstrates that T dependence or T independence is not necessarily a fundamental characteristic of the antigen, but rather reflects the way the antigen is presented.

According to current immunological dogma, cells that express class II MHC are responsible for initial presentation of antigen to helper T lymphocytes (Th) during induction of immunity. Helper T cells are characterized by the presence of CD4 molecules on the cell surface. The CD4 molecule is part of an immunoadhesion receptor complex that specifically recognizes the

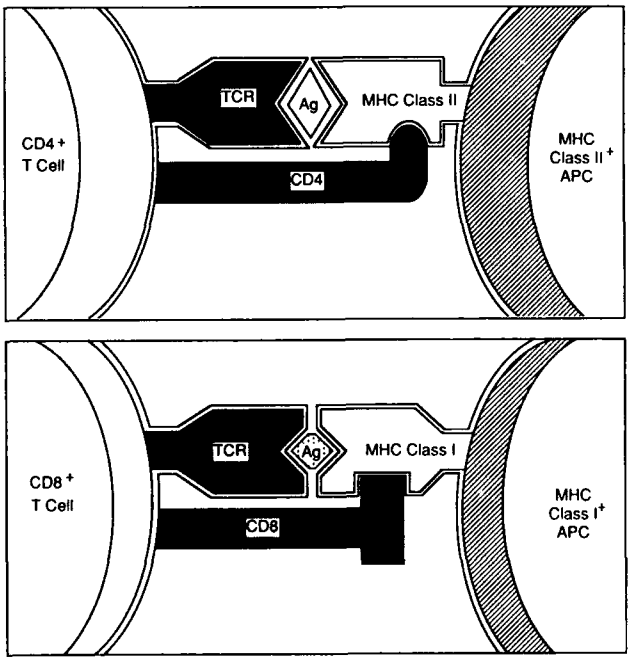

Fig. 1. Adhesion complex leading to presentation of antigen to T lymphocytes by antigen presenting cells. Abbreviations: APC, antigen presenting cell; Ag, antigen, MHC, major histocompability complex; TCR, T cell receptor. See text for details. Redrawn from Ref. 6.

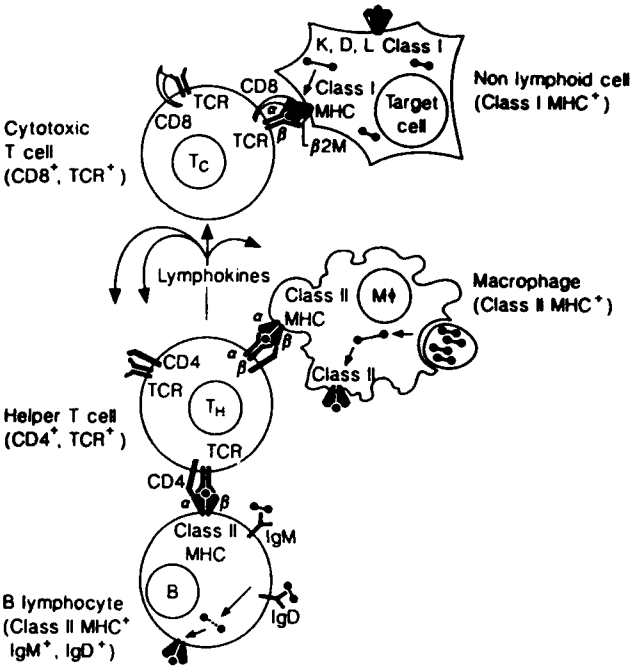

Fig. 2. A simplified presentation of the cellular interactions leading to virus-specific immune responses. Abbreviations: B, B cell, a lymphocyte that carries immunoglobulin (Ig) molecules on its surface; lymphokines, small regulatory proteins that are produced by lymphocytes and affect the activities of other cells of the immune system; Mø, macrophage; Tc, cytotoxic T lymphocyte; helper T lymphocyte; TCR, T cell receptor. See text for futher details. Reproduced with permission from Ref. 18.

combination of the autologous class II MHC and the particular peptide antigen or antigen fragment bound to the MHC on the surface of the APC (Fig. 2). The binding of the receptor on the $CD4^+$ Th to the peptide-MHC II complex on the APC triggers a 'conversation' between the Th and the APC in the form of secretion of a complex array of mediators and lymphokines, including membrane-bound and secreted IL-1 from macrophages and interferon-γ (γ-IFN) and interleukin-2 (IL-2) from T cells. The conversation between activated macrophage APCs and Th results in the initiation of a process of recruitment of numerous types of specific progenitor T lymphocytes and B lymphocytes leading to proliferation of specific B or T cell clones expressing specific immunity (e.g., antibody production or CTLs) against the antigen that was originally taken up by the APC (Fig. 2).

II-B. Influence of phospholipids on activities of MHC molecules

MHC molecules have a hydrophobic region that can span lipid bilayer membranes and it might be expected that they could be influenced by membrane lipid composition. Several studies have shown that immunological presentation involving class II MHC antigens

can be strongly diminished or blocked by treatment of APCs with phospholipase A_2 or phospholipase C [19–23]. This indicates that phospholipids on the APC may be critically important for functional activity of the class II MHC molecules. In studies described below, liposomes have been shown to have utility in demonstrating the physical and chemical factors that regulate reconstitution of MHC molecules in a form that is functionally active at the surface of lipid bilayer membranes.

II-C. Reconstitution of MHC molecules by liposomes

MHC molecules are poorly soluble amphipathic substances and are usually isolated from cells by using detergents. Utilization of purified MHC molecules in immunological presentation studies is influenced by at least two factors: the detergents used to isolate MHC molecules are potentially toxic to lymphocytes and other cells in the immune system in the amounts required for biological studies; and the conformation of the MHC molecule in the membrane may influence the immunological presentation role of the MHC or the functioning of the MHC molecule itself as an antigen.

Liposomes have been widely used to reconstitute MHC molecules (Refs. 24–26; reviewed in Refs. 27,28). Reconstitution of MHC into liposomes is most often achieved by techniques of detergent dialysis or gel filtration (methods for removal of detergent are reviewed in Ref. 29). For example, a dialyzable detergent such as deoxycholate or octylglucoside is used to solubilize both the lipids and the MHC, and upon removal of the detergent by dialysis the MHC is reconstituted into liposomes that are then formed spontaneously. In at least one case the affinity of the MHC for reconstitution into liposomes was so great that a liposome reconstitution procedure was actually suggested as a method for purifying the MHC [30]. An interesting method for separating a nondialyzable detergent (Triton X-100) from liposomes with polystyrene beads has been applied to reconstitution of MHC into liposomes [31].

As noted earlier, MHC molecules have a hydrophobic sequence, and in the absence of the hydrophobic sequence association with liposomes does not occur [32]. The MHC incorporates assymetically into liposomes, and it has been presumed that the MHC is in a transmembrane form. Based on enzymatic and serological data, MHC reconstituted into liposomes was found to be at least partly or fully in a 'right side out' configuration similar to that found in cells [30,33–37]. It has been pointed out, however, that the transmembrane conformation and orientation of MHC in liposomes has never been definitively established and a variety of liposomal configurations of MHC are possible [38]. Reconstituted H-2 MHC in liposomes does retain biological activity, and it has been concluded that reconstituted liposomal MHC need not necessarily reproduce the exact transmembrane configuration that is present in the plasma membrane of cells in order to serve as a model for immunological presentation events.

The influence of phospholipid type on the reconstitution of radioiodinated lysozyme or ovalbumin peptides with I-A^d molecules has been examined in detail [39]. Phosphatidylcholine, phosphatidylserine, phosphatidylinositol, and cardiolipin markedly enhanced binding, but phosphatidylethanolamine and sphingomyelin had no effect. Apparently the headgroup was more important for influencing reconstitution than the *sn*-2 fatty acid since lyso derivatives of reactive phospholipids were also reactive. However, from the empirical data obtained in the above study it was not possible to make any other generalizations relating to charge, polar headgroup type, or fatty acid composition. As is often the case in reconstitution studies, the physical and chemical factors of lipids or detergents that lead to optimization of reconstitution of hydrophobic proteins are complex and not yet completely understood. Patterns of results such as those described above may prove to be different with other types of MHC-peptide combinations.

As discussed in sections below, liposomal antigen can be combined with class II MHC at the surfaces of APCs. This process can cause presentation to T lymphocytes, thereby leading to the formation of antibodies and CTLs.

II-D. Interactions of liposomes with antigen presenting cells

One of the better known observations in the cell biology of liposomes is that liposomes are taken up avidly by macrophages both in vitro [40–47], and in vivo [48–54]. The interaction of liposomes with macrophages has been referred to as 'the macrophage connection' [55], and the presumed role of macrophages as APCs for liposomes has served as the major rationale for using liposomes as carriers of vaccines [9,56–58].

The importance of macrophages in the immune response to liposomal antigens was suggested by in vivo studies in which macrophages (but not B lymphocytes or dendritic cells) were depleted in animals either by injection of carrageenan [59] or liposomes containing a cytotoxic agent (dichloromethylene diphosphonate) [60,61]. These treatments resulted in severe suppression of immune responses to liposomal antigens. Recovery of the immune response occurred in parallel with reappearance of the macrophages [60,61].

In a series of elegant in vitro experiments Dal Monte and Szoka demonstrated that liposome-encapsulated antigen could be immunologically presented to T cells

by peritoneal exudate cells (containing both B and T cells and macrophages) after incubation of liposomes with the peritoneal exudate cells, but presentation did not occur after incubation of the liposomes with cells from a B cell tumor line [62]. In these experiments presentation was determined by IL-2 secretion from hybridoma helper T cells. It was subsequently found that when the antigen was covalently coupled to the surface of the liposomes the antigen could be presented, albeit inefficiently, by the B cell tumor [63].

Recently, it was reported that ovalbumin encapsulated in pH-sensitive liposomes that were delivered to dendritic cells could induce primary CTL responses in vitro [64,65]. Liposomes that were pH-insensitive did not induce CTLs. Delivery of the pH-sensitive liposomes to cultured macrophages also did not induce CTLs. Macrophages and dendritic cells that were subsequently isolated from animals immunized with pH-sensitive liposomes containing ovalbumin antigen served as APCs in vitro, but the dendritic cells had more potent activity than macrophages for serving as APCs for induction of CTLs.

Based on all of the above evidence, it appears that although macrophages are probably the most important element in the processing of liposomal antigens, presentation by B cells and dendritic cells is not necessarily excluded.

II-D.1. Intracellular fate of liposomal antigen

It has been reported that antigens introduced directly into the cytoplasm of cells can enter the class I pathway of antigen processing and presentation [66]. A concept was therefore developed for producing liposomes that were pH-sensitive and targeted intracellularly to the cytoplasm. This concept was derived from the observation that certain weakly acidic liposome-encapsulated molecules could escape into the cytoplasm of cells after endocytosis of the liposomes [67]. These pH-sensitive liposomes were originally designed for the purpose of delivering liposome-encapsulated endocytosed macromolecules and drugs to the cytoplasm of cells [68,69]. Liposomes that promote delivery of antigen to a cytoplasmic location might

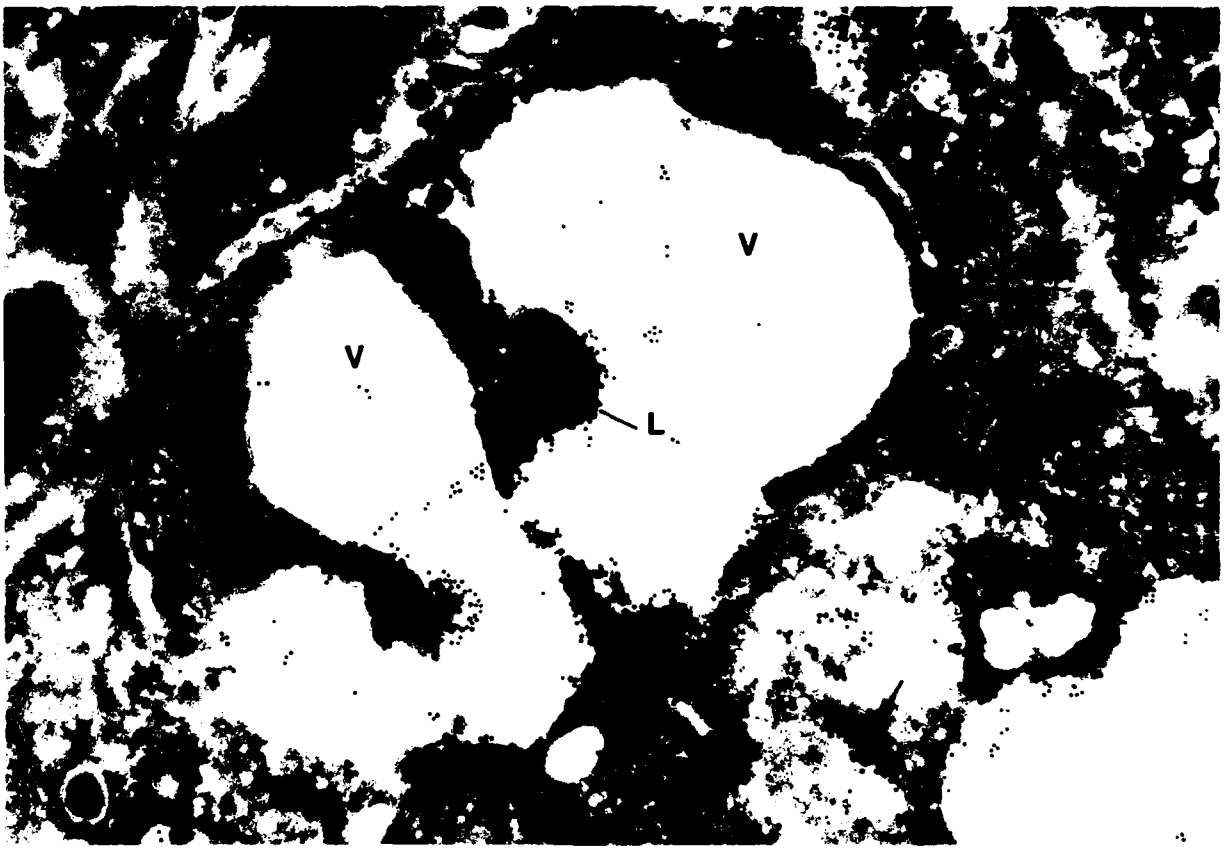

Fig. 3. Immunogold electron microscopy of cultured bone marrow-derived macrophages 6 h after pyhagocytosis of liposomes containing malaria antigen ($R32NS1_{81}$). The malaria antigen was detected by a specific monoclonal antibody to the antigen followed by treatment with gold-labelled second antibody. V, vacuole; L, liposome containing antigen. Four arrows indicate examples of locations of cytoplasmic antigen. From Ref. 75; see Ref. 47 for further details.

therefore have properties that would favor induction of class I MHC-restricted CTLs [70,71]. However, it has been recently observed, as described later (section II-F.2) that CTLs are generated in vivo both by pH-sensitive and pH-insensitive liposomes [72].

It should be noted that from an immunological standpoint an initial immune response leading to generation of CD8 + (class I MHC-restricted) CTLs can involve interactions of antigen with macrophages as APCs [73], and can also involve the participation of CD4 + Th in the process of induction of CTLs [74]. In a recent study the intracellular fate of a recombinant malarial antigen encapsulated in pH-insensitive liposomes was investigated by the technique of immunogold electron microscopy [47]. After phagocytosis of the liposomes by bone marrow-derived macrophages, liposomes containing encapsulated antigen accumulated in large intracytoplasmic vacuoles, and within the vacuoles the liposomes were often closely associated with the vacuolar membrane (Fig. 3). It can also be seen, based on the distribution of gold particles, that the antigenic contents of some of the liposomes in the vacuoles were being released in relatively large amounts into the cytoplasm of the macrophage. Despite the transport of antigen from the vacuoles to the cytoplasm, no clear liposomal structures per se were observed in the cytoplasm. Although well defined liposomes were not observed in the cytoplasm, upon close examination of the electron micrographs some of the areas around accumulations of cytoplasmic gold particles seem to be slightly more electron dense than the surrounding cytoplasm, possibly indicating the presence of some amorphous lipid accumulation.

It is possible that the ability of liposomes to serve as carriers both for generation of humoral immune responses (class II MHC-restricted) and for generation of CTLs (class I MHC-restricted) may occur because of the unique ability of liposomes to deliver liposomal antigens both to intracellular acidified vacuoles (where class II molecules are located) and to cytoplasm (where access to class I molecules is possible). By using a monoclonal antibody specific for the liposomal antigen, the appearance of the antigen on the surface of the macrophages was also observed within 15 min, after initial incubation of liposomes with the cells, and continuous expression of the antigen on the macrophage surface occurred for approximately 24 h, probably due to slow release of the antigen from the liposomes [47]. Presentation of the liposomal NANP-containing malaria antigen by bone marrow-derived macrophages to NANP-specific T lymphocytes after phagocytosis of the liposomes has recently been demonstrated [76]. This supports the concept that the liposomal antigen illustrated in Fig. 3 was participating in an antigen presentation pathway. In the latter study it was of further interest that liposomal lipid A served as an intracellular adjuvant to enhance the presentation of the liposomal antigen by the macrophages [76].

II-E. Antigen presentation by reconstituted liposomes

Liposomes and other model membranes reconstituted with MHC and immunogenic proteins or peptides have been studied on many different levels. Numerous groups of investigators have used model membranes to mimic antigen-specific MHC II-restricted presentation to helper T cells [30,62,63,76–89] and to model or elicit specific CTLs in vitro [24,26,28,90–99]. Biophysical aspects of T cell recognition of antigen on supported planar lipid membranes have been reviewed by Watts and McConnell [100].

From a biochemical and biophysical standpoint an ideal goal in presentation studies might be to utilize liposomes as 'artificial cells' that could substitute for APCs. Claims of success have been made in the development of 'antigen-presenting liposomes' and 'artificial macrophages' (see section II-G), and these studies have become particularly more interesting with the development of highly defined lymphoid cells and cell lines to which reconstituted liposomal antigens can be presented. Background and supplementary information can be obtained from an earlier review [27].

Mixed lymphocyte populations (for example, splenic cell cultures) from immunized donor animals, or mixed lymphocyte populations that have been immunized in vitro, have often been used as signal cells that respond to liposomal antigen presentation in vitro. Numerous studies have demonstrated the occurrence of antigen-specific MHC-restricted proliferation of such cells upon incubation with liposomes [27]. Mixed populations of cells do contain numerous APCs, including B lymphocytes and adherent cells (macrophages), and it is presumed that the presentation process of mixed cell populations involves those APCs [101,102].

II-F. Generation of cytotoxic lymphocytes by reconstituted liposomes

II-F.1. In vitro studies

A type of antigen presentation that occurs in the effector phase of cell-mediated immunity is manifested by the killing of target cells by cytotoxic lymphocytes (CTLs). In this case the target cell itself serves as an APC for surface presentation of its own antigen with class I MHC. The antigen-class I MHC complex is displayed on the surface of the affected cell. A receptor complex on the CTL that usually includes CD8 as part of the receptor specifically recognizes the antigen-class I MHC complex on the target cell (Figs. 1 and 2), and when binding occurs via the receptor the CTL can kill the target cell.

The target cell antigens that are presented to CTLs most often are antigens that were originally derived from intracellular rather than extracellular sources, and they include viruses, parasites, and self-antigens such as cancer antigens. In an exception to this, it has also been shown that direct introduction of purified antigen into the cytoplasm of cells by osmotic lysis of pinosomes promotes class I-restricted presentation of the antigen with associated induction of CTLs [66]. However, as pointed out earlier (section II-D.1), CTLs to liposome-encapsulated antigen are generated both by pH-insensitive liposomes that are thought to be processed mainly by acidified intracellular vacuoles and pH-sensitive liposomes that release their contents directly into the cytoplasm [72].

Cytotoxic lymphocytes can be generated in vitro by incubating mixed exudate cells (containing macrophages and lymphocytes) with foreign cells (allogeneic cells from different genetic stains, or tumor cells) or with viruses. Modeling of this phenomenon has been extensively investigated with liposomes [27]. Target cells that are specifically killed by CTLs may consist of foreign cells containing tumor antigens or containing genetically different (allogeneic or xenogeneic) MHC molecules that serve as antigens. Target cells can also be obtained by coating or infecting cultured cells with viruses or viral proteins. Cytotoxic lymphocytes may be induced in vitro either by 'priming' mixed populations of splenic cells with an appropriate liposomal antigenic stimulus, or they may be induced by incubating previously primed cells with an antigenic stimulus to produce a 'secondary' immune response.

Reconstituted liposomes that stimulate secondary CTLs in vitro have been made with allogenic MHC antigens, xenogeneic MHC antigens, viral antigens, and tumor antigens (Table I). Among the many issues that have been addressed in the course of in vitro stimulation of CTLs by liposome-reconstituted antigens have been the following: induction of primary immunity vs secondary proliferation of primed lymphocytes; role of adherent cells (macrophages) as antigen presenting cells; requirement for the simultaneous presence of MHC molecules together with antigen molecules in the same liposomes; requirement for additional signals (such as IL-2); orientation of reconstituted antigen; role of carbohydrate constituents of antigen; and influence of membrane lipid composition.

An early study demonstrated that plasma membrane fractions were 20-fold less effective than intact cells for stimulating a primary CTL response [117]. Nonetheless, in vitro primary CTL responses with liposome-reconstituted antigens have been described by two laboratories [94,115,118,119]. As might be expected, adherent cells were necessary for generation of primary in vitro murine CTL immunity against a xenoantigen (human colon tumor antigen) [115].

Perhaps most interestingly, surface antigen appeared to be important for generation of a primary CTL immune response, and removal of liposomal surface antigen by trypsin treatment significantly reduced the induction of the response [115]. Previous claims have been made that liposomal surface antigen, but not encapsulated antigen, can induce antibody formation; however, upon further examination, it was found that it was not possible to generalize the role of surface antigen, and encapsulated antigen and surface antigen have both shown to be immunogenic (this controversy and its resolution are reviewed in Ref. 9). It is possible that the relative immunogenicity of surface vs encapsulated antigen may depend on the particular antigen or model immune system being employed.

TABLE I

Liposomal antigens used for in vitro induction of cytotoxic T lymphocytes

Liposomal antigen used for immunization	Reference
Allogeneic MHC	
Murine H-2-MHC alloantigen	[103,104]
Purified murine class I H-2 MHC	[28,37,91,92,105–107]
Xenogeneic MHC	
Purified human HLA xenoantigens	[28,108–110]
Viral antigens	
Sendai virus protein + syngeneic H-2 MHC	[111,104]
Hemagglutinin/neuraminidase glycoprotein from sendai virus	[112]
Vesicular stomatitis virus G glycoprotein	[113]
Herpes simplex virus type 1 antigen + syngeneic H-2 MHC	[90]
Whole influenza virus	[114]
Tumor antigens	
Human colon tumor cell antigens	[94,115]
Moloney murine sarcoma virus-induced tumor membrane antigen	[116]

II-F.2. In vivo studies

In an early unsuccessful study induction of CTLs in vivo was not achieved under conditions in which herpes simplex virus type 1 (HSV-1) antigens were incorporated into liposomes [120]. Although infectious HSV-1 itself induced an excellent immune response, liposome-encapsulated HSV-1 antigen did not induce CTLs even when the liposomes contained lipid A as an adjuvant. The first indication, albeit indirect, that CTLs might be possible to obtain with a liposomal antigen came from a report that an HSV type 2 peptide conjugated to palmitic acid inserted into liposomes could cause protection against HSV type 2 infection [98]. The protection was transferred by T cells but not by serum.

In a later study, virosomes consisting of liposomes containing influenza virus hemagglutinin and neuraminidase (f-NANA) and also containing lipophilic muramyl dipeptide (B30-MDP) induced specific CTLs in vivo, but immunization with f-HANA alone did not induce CTLs [121]. Liposome-encapsulated human lymphotropic virus type 1 (HTLV-1) did not induce CTLs, but CTLs were induced when the liposomes were coated with a mannan derivative [122].

Recently, three laboratories have demonstrated that specific CD8 + (MHC class I-restricted) CTLs can be readily induced in vivo with at least three different liposome-encapsulated soluble proteins, ovalbumin [72,123], β-galactosidase [72], and a recombinant human malaria antigen [124]. Lysophosphatidylcholine, a compound that was thought to have fusogenic activity, was included as a liposomal constituent in one study, and CTLs were observed irrespective of neutral, negative, or positive liposomal surface charge [123]. Induction of CTLs was also observed both with highly unsaturated pH-sensitive liposomes (dioleoyl phosphatidylethanolamine/1,2-dioleoyl-*sn*-3-succinyl glycerol) and with pH-sensitive liposomes (dioleoyl phosphatidylcholine/phosphatidylserine/cholesterol) [72].

II-G. Liposomes as models of macrophages: 'cell-free' antigen presentation

Cell-free antigen presentation by lipid model membranes in the absence of APCs has been achieved in parallel lines of investigation, either with the use of supported planar membranes [77,78,83,85,86,95,100] or with the use of liposomes [62,63,79–82,125,126]. Replacement of cells with model membranes has represented a major advance in elucidating the mechanism of antigen presentation.

An early report demonstrated that synthetic supported planar lipid membranes containing purified Ia (class II MHC) antigen could present a peptide digest of ovalbumin to a syngeneic ovalbumin-specific helper T cell hybridoma, but when the same material was included in liposomes presentation was not observed [77]. This observation implied that a fundamental difference might exist between the modeling of antigen presentation by planar membranes and liposomes. However, in a series of extraordinarily interesting experiments, Walden et al. devised cell-free synthetic liposomal systems in which undigested native antigens were presented to cloned T cells and T cell hybridomas in the absence of APCs [79–82]. By analogy with APCs, liposomes that present antigen in the absence of APCs have been referred to as 'antigen presenting liposomes' [55].

In the system devised by Walden et al., antigen-presenting liposomes were prepared containing native protein antigen (ovalbumin or bovine insulin) covalently coupled to the surface liposomal lipids. The conjugation of protein to the lipids was accomplished after activation of protein and liposomal lipids with a heterobifunctional reagent *N*-succinimidyl 3(-2-pyridyldithio)propionate (SPDP) [127,128]. Purified detergent-solubilized class II MHC molecules were also inserted into the liposomes by reconstitution. With these liposomes that contained both antigen and MHC molecules, MHC-restricted antigen-specific presentation was demonstrated by proliferation of an antigen-specific T cell clone or IL-2 secretion by an antigen-specific T cell hybridoma in the absence of APCs [79]. Presentation only occurred when liposomes contained both the appropriate MHC molecules and the original priming antigen. The authors concluded therefore that uptake of liposomes by undetected residual APCs did not occur and also that degradation of native antigen to smaller peptide fragments was not required for antigen presentation.

Further studies on the liposomal lipid requirement showed that reconstituted liposomes containing only dipalmitoyl phosphatidylethanolamine (DPPE) stimulated T cells 20 to 25-fold more than highly fluid liposomes containing dipalmitoyl phosphatidylcholine/dioleoyl phosphatidylcholine/oleoylpalmitoyl phosphatidylcholine/DPPE (5:1:3:1) [80]. The conclusion was made that the degree of presentation was more dependent on the types of reconstituted lipids than on the degree of fatty acid unsaturation. However, it should be pointed out that the relative surface areas of the different liposomes that were utilized were not held constant, and surface area could certainly be viewed as an important variable in the process of antigen presentation.

Cell-free MHC-restricted presentation of liposomal antigen was also dependent on the density of the surface bound antigen. With certain T cell lines, when the density of bound antigen on the liposomes was sufficiently high, presentation occurred even in the complete absence of MHC molecules [80]. This latter novel observation was confirmed by Dal Monte and Szoka [63] but it was also viewed skeptically in a short critique by Murphy [129]. It was proposed by Murphy that class II MHC molecules might have been 'shed' by contaminating cells and incorporated into liposomes, and that this theoretical possibility could be tested by the inclusion of antibodies to class II molecules in the cultures to block any possible effects that might have required class II MHC. In response to this criticism, antibodies against both class II MHC molecules and against another bound liposomal antigen, pigeon cytochrome *c* (PCC), were then tested by Walden et al. for inhibitory properties against stimulation of IL-2 by an antigen-specific MHC-restricted T cell hybridoma [81]. The results showed that the MHC-restricted response of the hybridoma was specifically blocked by

both anti-MHC and anti-PCC monoclonal antibodies; in contrast, MHC-independent stimulation of the hybridoma by liposomes containing a high epitope density of PCC was blocked only by anti-PCC [81]. Despite the growing evidence that antigen presentation can be achieved with very high epitope densities of liposomal antigen in the absence of MHC molecules, further studies probably will be required in order to achieve complete acceptance because of the novelty of this concept.

In a further interesting twist to the above studies, antigen-specific monoclonal antibodies (anti-lactic dehydrogenase and anti-PCC) were conjugated to MHC class II-reconstituted liposomes, and MHC-restricted stimulation of a T cell clone or hybridoma was then induced in the presence of the appropriate soluble antigen [82]. This latter experiment dispelled the notion that the antigen was inappropriately altered during chemical conjugation to the liposomes. It also provided a model for antigen presentation by cell surface-bound (e.g., B lymphocyte-bound) antibodies. It is interesting to note incidentally that covalently-bound Fab fragments of anti-IgM antibodies on liposomes have been used to cross-link IgM antibodies on the surface of B cells. This latter technique was used as a method to model mitogenesis of B cells that occurs upon binding of antigen to antibodies on the surface of B cells [130].

The basic elements of the Walden et al. findings have recently been confirmed by two other laboratories [63,125,126]. Presentation of surface-bound molecules lacking MHC antigen to helper T cells by liposomes in a cell-free (APC-free) system was also confirmed. In the study by Dal Monte and Szoka [63], the antigen consisted either of native PCC or a synthetic peptide representing a T cell determinant of PCC. Thiolation of the protein prior to binding to liposomes was accomplished either by use of SPDP or alternatively by use of 2-iminothiolane. Helper T cells that served as the target for presentation consisted of a PCC-specific T cell hybridoma, and activity induced by the antigen-presenting liposomes was determined by IL-2 secretion. When the free (nonliposomal) antigens were incubated with glutaraldehyde-fixed APCs (peritoneal exudate cells) the PCC was not presented by the APCs to T cells, but the T cell peptide determinant was presented. This observation was consistent with a requirement for degradation of the free antigen by APCs. In contrast, after anchoring the antigens to the liposomal surface both PCC and the T cell determinant were presented by the APCs. It was concluded that processing (degradation) of liposomal protein was not required for presentation to T cells. Moreover, in confirmation of the work of Walden et al., in the absence of APCs a low level of direct stimulation of T cells by high concentrations of liposomes containing antigen but lacking MHC molecules was also demonstrated. It was therefore concluded that under some circumstances high concentrations of liposomal antigen can bypass the requirement for participation of MHC molecules.

II-H. Role of mediators

As a corollary to the above cell-free antigen presentation experiments it was concluded that APC-derived mediators were not required in order to achieve antigen presentation. However, macrophages are known to secrete interleukin (IL-1) as part of the 'conversation' with helper T cells in the process of antigen presentation. A membrane form of IL-1 from monocytes exhibited mitogenic activity after incorporation in liposomes [131] and so-called 'synthetic macrophages' consisting of liposomes containing reconstituted class II MHC, IL-1, and bound native antigen were shown to have enhanced MHC-restricted antigen-specific presentation properties to a T cell clone [125,126].

III. Immune responses to liposomal phospholipids and cholesterol

III-A. Historical background

As noted in the Introduction, there is an extensive body of research, that traces its origins as far back as 1906, that suggests that phospholipids themselves, and even cholesterol, can be immunogenic [16,132,133]. The topic of 'phospholipid-binding antibodies', including the preparation, properties, and clinical implications of such antibodies, was the subject of a recent excellent multiauthored book [134].

III-B. Presentation of lipid antigens

All of the theories and models of antigen presentation that have been developed to date have been based on studies involving the immune behavior of peptide and protein antigens. There is essentially no literature dealing with presentation of lipid antigens. In fact, to my knowledge there has not yet been any demonstration of a T cell that specifically recognizes a phospholipid or cholesterol epitope. Although I presume that T cells having specificities for such epitopes probably do exist, this review will cover only the question of the existence of immune responses to phospholipids and cholesterol.

From a historical standpoint at least two important events have greatly shaped the thinking about lipids as antigens. The first was the evolution of the concept of haptens, a term proposed by Karl Landsteiner [12]. Although Landsteiner extensively studied lecithin and cholesterol as antigens, he ultimately developed the

view that "...there exist two systems of species specificity in the animal kingdom, the specificity of proteins and that of cell haptens", and he included phospholipids and cholesterol in the category of haptens [12]. Landsteiner therefore concluded that although antibodies apparently could bind to lipids, the lipids themselves could stimulate an immune reaction only when they were attached to a large carrier molecule. This conclusion promulgated by Landsteiner subsequently led to the widespread, but incorrect, view that proteins are the only antigens that can exist in Nature, and that lipids in general, and particularly phospholipids, are not immunogenic by themselves [13].

The second important historical event in the development of lipid immunology, an event that influenced thinking about lipid antigens in a more practical direction, was the necessity that evolved some years ago for commercial development of immunoassays for steroid hormones. Because of the ease with which antibodies to steroid hormones can be induced by immunizing with carrier-steroid conjugates, hundreds of useful and highly specific immunoassays for steroid hormones have been devised [135,136]. Although this latter development supported the view that steroids could serve as haptens, it also firmly established the concept that highly specific antibodies, even monoclonal antibodies, can be produced that specifically recognize epitopes on steroidal structures derived from cholesterol. The historical aspects, including the various controversies, of lipid immunology have been extensively reviewed elsewhere [16].

The passage of time has resulted in the unfolding of much greater complexity, and a higher level of sophistication, in the understanding of the three dimensional molecular architecture of particulate arrays of lipid antigens than was previously possible, and this has changed our views of lipids as antigens. The development of liposomes as models for effector mechanisms of immune reactions [15,137], when combined with the discovery of the immunogenicity of liposomal phosphatidylcholine and other liposomal phospholipids [16,132,138] and the production of monoclonal antibodies to liposomal cholesterol [139] (see Ref. 133 for detailed review of antibodies to cholesterol), has provided new tools for examining the interactions of lipids with the various afferent limbs of the immune system.

The mechanisms by which lipid antigens are handled by the immune system are unknown. Although it is reasonable to presume that APCs participate in the immune response to phospholipids and cholesterol, it would seem unlikely that degradation of lipid antigens by APCs would be required as part of the process. In view of the ability of liposomes containing high epitope densities of antigen to escape MHC restriction during presentation (see section II-G) it seems reasonable to speculate that under certain conditions presentation of liposomal phospholipids and cholesterol might also avoid MHC restriction.

III-C. Antibodies to bromelin-treated erythrocytes: a model of anti-lipid autoimmunity

The production of antibodies to phospholipids and cholesterol obviously represents a form of autoimmunity. Immunologists have devised a number of widely used murine and human models for studying autoimmunity, one of which consists of autoantibodies against bromelin-treated erythrocytes. In this model the antigen in the bromelin-treated erythrocytes that binds the autoantibodies has now been identified as phosphatidylcholine (refs. 140–145; see Refs. 16,146,147 for reviews). Bromelin is a proteolytic enzyme, and it is presumed that treatment of the erythrocytes with bromelin exposes the underlying cryptic phosphatidylcholine antigen and allows the binding of antibodies. In the bromelin-treated erythrocyte model the anti-phosphatidylcholine antibodies that are studied either occur naturally [140,142–144,148] or are induced by injecting animals with lipopolysaccharide (LPS) from Gram-negative bacteria [141,149]. The endotoxic region of LPS consists of lipid A, and it has been shown that lipid A alone induces autoantibodies to mouse erythrocytes [150].

In an independent line of research, liposomes containing lipid A have also been used as immunogenic particles for inducing 'anti-liposome' antibodies that have specificity against individual liposomal phospholipids (e.g., phosphatidylcholine, phosphatidylserine, phosphatidylinositol phosphate, etc.) [16,132], or even antibodies that have specificity against liposomal cholesterol [16,132,133]. Naturally-occurring antibodies to liposomal phospholipids and cholesterol are widespread in normal sera from animals and humans [16,132,133,148–153]. Remarkable similarities exist between the anti-liposome antibody model and the model employing autoantibodies that react with bromelin-treated erythrocytes, and this has led to the suggestion that the two models are different manifestations of the same underlying phenomenon [16,146,147].

III-D. Immunogenicity of phospholipids and cholesterol

Polyclonal antisera and monoclonal antibodies have been produced by a variety of methods against numerous phospholipids, and also against cholesterol [139], and these topics have been the subjects of numerous reviews [13–16,132,133,154,155]. A classic method described by Inoue and Nojima demonstrated that antibodies to acidic phospholipids could be produced in rabbits by intravenous injection of a mixture of cardiolipin, lecithin, cholesterol, and a carrier protein con-

sisting of methylated bovine serum albumin [156]. Other methods utilizing liposomes or other nonproteinaceous carriers are described in sections below.

Nearly all of the methods for inducing anti-phospholipid antibodies with protein-free formulations that have been utilized to date have employed an adjuvant, most commonly lipid A (see below). Recently, Rauch and Janoff reported the unique and important observation that immunization of mice with phosphatidylethanolamine alone, under conditions in which the phospholipid was in the hexagonal II phase but not under conditions in which it was in the lamellar bilayer phase, resulted in the induction of anti-phospholipid antibodies [157]. The antibodies that were induced by this technique were reactive with phosphatidylethanolamine and cross-reacted with cardiolipin.

III-E. Immunosuppressive properties of lipids

It has been noted that certain lipid formulations can have immunosuppressive properties. Antiserum, or even monoclonal antibodies, against phosphatidylserine (or phosphatidic acid) can be readily obtained in titers that are equivalent to titers achieved against other phospholipids by using a protocol that includes multiple immunizations [158–160]. However, it was also observed that after only a single primary immunization an immune response did not occur against phosphatidylserine or phosphatidic acid even though various other phospholipid formulations invariably induce a primary immune response after a single injection [160]. This therefore represents immunosuppression that is manifested primarily during primary immunization with an antigen. In another example of immunosuppression, it was noted in the course of immunizing animals with liposomes containing lipid A for production of antibodies either to protein or lipid antigens, that when prostaglandin E_2 or thromboxane B_2 was included in the liposomes the primary immune responses that normally occurred against both protein and lipid antigens in liposomes were strongly suppressed [161].

The primary immune response, which presumably relies mainly or exclusively on the initial processing and presentation of liposomal protein and lipid antigens by macrophages, apparently can be adversely influenced by certain lipid formulations. The mechanisms of the immunosuppressive effects of these lipids are not yet fully understood, but they might be related either to direct effects on macrophage membranes or to an influence on intracellular lipid metabolism. A suppressive effect of phosphatidylserine on the activation of macrophages by interferon-γ has been described [162,163]. The latter suppressive effect was thought to be related to changes of intracellular phospholipid metabolism through effects on phospholipase A_2 [163].

III-F. Antibodies to liposomal phospholipids

In 1979 it was reported that injection of liposomes containing lipid A into rabbits resulted in the production of anti-liposome antibodies that reacted with phosphocholine, phosphatidylcholine, sphingomyelin, and lipid A [138]. The antibodies were detected by complement-dependent immune damage to liposomes, and the interesting observation was made that the process was often enhanced when a glycosphingolipid, or even ceramide alone, was included in the liposomes [164,165]. The enhancing ability of ceramide has also been observed with monoclonal antibodies to phospholipids [166] (see Section III-H) and with complement-dependent damage to liposomes containing cholesterol [167]. Balanced against the enhancing effect of ceramide is the observation that when glycosphingolipids are present in high epitope densities in the liposomal bilayer the oligosaccharide headgroups of the glycolipids can sometimes interfere, either by steric hindrance or by charge interference, with antibody binding to the underlying lipids at the surface of the liposomal bilayer [165].

III-G. Anti-liposome antibodies in immune defective mice

Studies in mice (nude mice) that are genetically deficient in T lymphocytes demonstrated that liposomal hapten-phospholipid conjugates [168] or liposomal phospholipids themselves [169] are T-independent antigens in mice. Furthermore, more detailed murine genetic studies also suggested that the same liposomal lipid antigens are T-independent antigens when present in liposomes that contain lipid A. The latter conclusion is based on the ability of F_1 male progeny of immunologically-defective CBA/N mice to produce antibodies to the liposomal lipids [169,170]. In contrast to this, liposome-encapsulated proteins are T cell-dependent antigens [171].

T-independent antigens normally induce IgM but not IgG antibodies, and this was indeed observed, albeit after only a single injection, with liposomal phospholipids in immune-defective mice [169]. If such genetic studies in mice have relevance to humans, it may be of interest to note that antibodies that are associated with thrombotic phenomena in the so-called anti-phospholipid antibody syndrome in humans primarily consist of very high titers of IgG antibodies when tested in the anti-cardiolipin antibody assay [134,172,173]. It has been proposed that hexagonal II phase phospholipids may be target antigens for thrombosis [154,155,157] and it would be useful to determine if such phospholipids are T independent antigens.

III-H. Monoclonal antibodies to liposomal phospholipids

Naturally-occurring antibodies to liposomal phospholipids are quite commonly found in normal

animal or human serum [16,174,175]. In a model system consisting of rabbits infected with *Trypanosoma rhodesiense*, antibodies against numerous liposomal specificities were detected and were associated with the infection [176]. The titers of anti-phospholipid antibodies in normal human sera are usually relatively low, but under certain circumstances it is now recognized that extremely high levels of antibodies, that may or may not have medical significance, have been observed in humans [134].

Because of the presence of naturally-occurring antibodies, and because of other difficulties that are often encountered in using polyclonal antiserum, murine monoclonal antibodies to liposomes were developed for experimental study [166,177]. Mouse monoclonal antibodies showed specificities that were quite similar to the specificities of rabbit polyclonal antisera. The availability of different monoclonal antibodies that showed exquisite specificity for the complete structures of different liposomes used for immunization, and less specificity for liposomes having subtly different structures, provided considerable support for the concept that specific antibodies were induced against the liposomes. This also provided evidence that the antibodies did not appear merely as the result of nonspecific polyclonal activation. As shown in Fig. 4, adsorption studies invariably demonstrated that the antibodies reacted most strongly with the liposomes that were used in the original immunization procedure. It can also be noted in Fig. 4 that the monoclonal antibodies reacted more strongly with liposomes containing a glycosphingolipid (galactosyl ceramide). This latter observation removed any possible doubt that the enhancement of anti-liposome reactivity caused by glycosphingolipids that was observed with polyclonal antiserum (see section III-F) was not due to naturally-occurring antibodies to the glycosphingolipid itself.

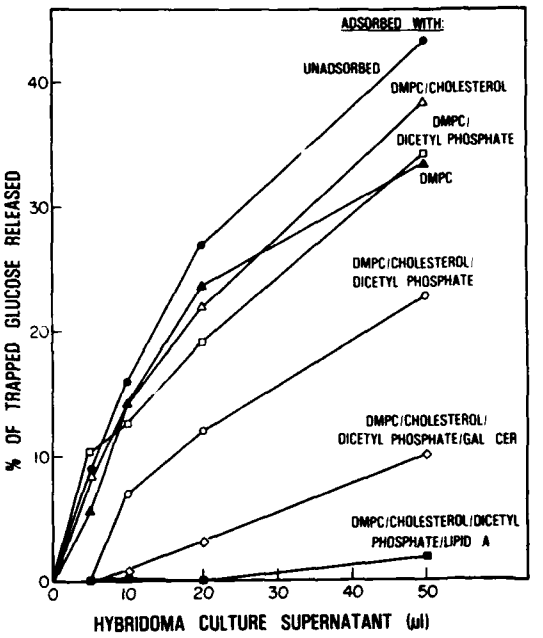

Fig. 4. Influence of liposomal lipid composition on binding of a murine monoclonal antibody to liposomes. The original immunizing liposomes contained dipalmitoyl phosphatidylcholine, cholesterol, dicetyl phosphate, and lipid A and the hybridoma clone secreting IgM antibody was selected by the ability to react with liposomes containing dimyristoyl phosphatidylcholine, cholesterol, and dicetyl phosphate. Binding was determined by loss of antibody activity after adsorption with the indicated liposomes. Adsorption of antibody resulted in decreased complement-dependent release of trapped glucose from liposomes containing dimyristoyl phosphatidylcholine, cholesterol, and dicetyl phosphate. From Ref. 166.

III-I. Specificities of antibodies to liposomal phospholipids

Anti-liposome antibodies induced by injecting liposomes containing lipid A into rabbits or mice invariably reacted most strongly with the liposome composition used for immunization. By manipulating the liposomal lipid composition, it has therefore been possible to develop rabbit polyclonal antiserum or mouse monoclonal antibodies that preferentially recognize various individual liposomal lipids, including phosphatidylcholine and sphingomyelin [138,166,178], phosphatidylsulfocholine [178], phosphatidylinositol phosphate [177], phosphatidylserine [160], and phosphatidic acid [160].

Although considerable cross-reactivity of antibodies to phospholipids often occurs with closely related phospholipids, exquisite specificities can also be observed. For example, immunization with liposomes containing dimyristoyl phosphatidylsulfocholine (DMPSC) resulted in production of antibodies both against DMPSC and cross-reacting antibodies to dimyristoyl phosphatidylcholine (DMPC) [178]. Upon adsorption of the DMPSC antiserum with DMPC liposomes, specific anti-DMPSC antibodies were obtained. In another example of antibody specificity, depending on the antibody examined, monoclonal antibodies to phosphatidylinositol phosphate (PIP) either did or did not cross-react with phosphatidylinositol (PI), cardiolipin, or phosphatidylserine [179]. It is also possible to produce monoclonal anti-PIP antibodies that either cross-react with PI but not with phosphatidylinositol diphosphate (PIP_2), or conversely, cross-react with PIP_2 but not with PI [177].

Because of the potential for cross-reactivity of anti-phospholipid antibodies with various phospholipids it is impossible to make broad generalizations about the specificities of such antibodies. Insights into the causes and specificities of cross-reactivities can be derived from hapten inhibition studies with monoclonal antibodies. To date, all of the antibodies studied have been inhibited by small soluble phosphorylated compounds. For example, inhibition is usually observed with phos-

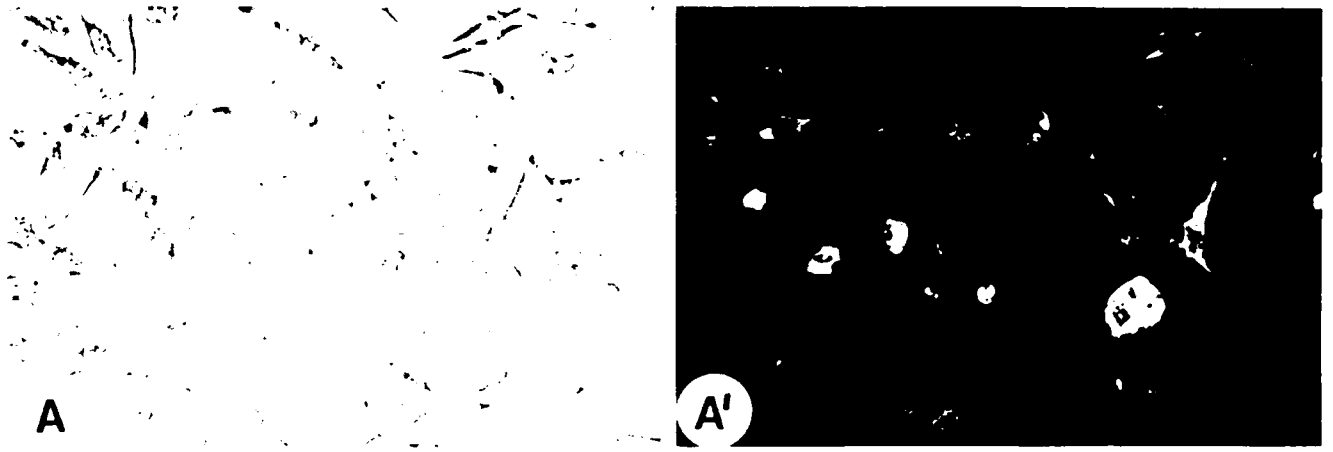

Fig. 5. Indirect immunofluorescence detection of macrophage surface-bound monoclonal anti-liposome antibody. (A) Phase-contrast micrograph of adherent murine macrophage culture incubated with antibody; (A') immunofluorescence micrograph of field under (A). From Ref. 183.

phocholine but not choline, and by inositol hexaphosphate but not inositol [166,177] The antibodies are also inhibited by ATP and, to lesser extent, by AMP. These observations have led to the conclusion that anti-liposome antibodies are directed against the entire mosaic pattern of lipid headgroups on the surface of the liposomes, but included within the antigen combining site of the antibody there exists a 'subsite' that specifically recognizes phosphate [132]. Another subsite that recognizes serine was also proposed based on hapten inhibition by serine of polyclonal antiserum against phosphatidylserine [160].

It has been previously pointed out that the structure of the antigenic site of cardiolipin (CL) is remarkably similar to the phosphorylated backbone of DNA [132,180,181] and cross-reactivity of anti-CL antiserum with DNA was demonstrated [181]. The phosphate-binding subsite that is present in antibodies to liposomal phospholipids, and the inhibition of the antibodies by a nucleotide (ATP), suggested the possibility that antibodies against liposomal phospholipids might also cross-react with DNA. Upon examination of four monoclonal antibodies to liposomal PIP and two monoclonal antibodies to liposomal cholesterol, strong cross-reactivities of three out of four of the anti-PIP antibodies were demonstrated with denatured DNA [182]. Binding to DNA was competitively inhibited by synthetic polynucleotides but not by nucleosides [182].

III-J. Binding of anti-phospholipid antibodies to cells

Monoclonal antibodies to liposomal phospholipids did not bind to mouse peritoneal macrophages that were kept in suspension, but large amounts of antibodies did bind to adherent macrophages [183] (Fig. 5). The binding was enhanced by treatment of the cells with trypsin, a result that would be expected if the phospholipids were cryptic antigens that were partially hidden under overlying cell protein. Antibody binding to the macrophages was inhibited by phospholipases C and D, but not by alkaline phosphatase or neuraminidase. It was therefore concluded that antibodies to liposomal phospholipids have the potential capacity to bind to cellular phospholipids but are usually inhibited from doing so by overlying cell surface protein. However, under some circumstances, such as after the occurrence of macrophage adherence or after damage to the cell surface protein integrity, antibody binding to cell surface phospholipids can occur [183].

III-K. Implications of antibodies to liposomal phospholipids for models of autoimmunity

The conclusion can be drawn from the studies in Sections III-H and III-I that antibodies to phospholipids that are generated by immunization with liposomes containing lipid A can exhibit considerable specificity for individual phospholipids. All of the antibodies that were studied had a subsite specificity that bound strongly to phosphate. Binding to phosphate can result in cross-reactivity with certain other phosphorylated molecules such as nucleotides and denatured DNA. The antibodies also reacted with cells that are treated with proteolytic enzymes. However, the specificities of the anti-liposomal phosphatidylcholine antibodies differ from the anti-phosphatidylcholine 'autoantibodies' that react with bromelin-treated erythrocytes. The latter antibodies reportedly have a strong choline-binding (trimethylammonium-binding) subsite [140], but the possibility that the latter antibodies also have a phosphate-binding subsite has not been examined. The data appear to be consistent overall with the likelihood that each type of antibody represents a different manifestation of a spectrum of similar antibody specificities. This later hypothesis has been discussed and reviewed elsewhere in detail [16,146,147].

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